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Primer-Dependent Synthesis of Covalently Linked Dimeric RNA Molecules by Poliovirus Replicase

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Poliovirus-specific RNA-dependent RNA polymerase (replicase, $3D^{pol}$) was purified from HeLa cells infected with poliovirus. The purified enzyme preparation contained two proteins of apparent molecular weights 63,000 and 35,000. The 63,000-M_r polypeptide was virus-specific RNA-dependent RNA polymerase, and the 35,000-M_r polypeptide was of host origin. Both polypeptides copurified through five column chromatographic steps. The purified enzyme preparation catalyzed synthesis of covalently linked dimeric RNA products from a poliovirion RNA template. This reaction was absolutely dependent on added oligo(U) primer, and the dimeric product appeared to be made of both plus- and minus-strand RNA molecules. Experiments with 5′ [^{32}P]oligo(U) primer and all four unlabeled nucleotides suggest that the viral replicase elongates the primer, copying the poliovirion RNA template (plus strand), and the newly synthesized minus strand snaps back on itself to generate a template-primer structure which is elongated by the replicase to form covalently linked dimeric RNA molecules. Kinetic studies showed that a partially purified preparation of poliovirus replicase contains a nuclease which can cleave the covalently linked dimeric RNA molecules, generating template-length RNA products.

Replication of the positive-polarity RNA genome of poliovirus proceeds through the formation of a complete negative-strand RNA which serves as the template for synthesis of progeny virion RNA molecules (4). In vitro synthesis of negative-strand RNA from a virion RNA template requires a 67,000-M_r host cell protein (host factor) and poliovirus-specific RNA-dependent RNA polymerase (replicase, p63, $3D^{pol}$) (5, 7–11, 17, 18, 20, 23). A synthetic RNA primer, oligo(U), can replace the host factor in the in vitro reaction. It is, therefore, believed that a host factor is needed for initiation of negative-strand RNA synthesis (6, 8, 9). In our laboratory we have been using a highly purified preparation of host factor and a partially purified preparation of poliovirus replicase to study in vitro replication of the viral RNA genome. A number of studies from our laboratory and those of others have shown that genome length copies of poliovirion RNA could be synthesized in vitro by this system (6, 7). In contrast, Young et al., using a highly purified preparation of replicase and a relatively crude preparation of host factor, reported in vitro synthesis of RNA molecules twice the size of the template RNA (25). Their results suggested that the in vitro synthesized negative-strand RNA was covalently linked to the input template RNA (plus strand). On the basis of these results, these researchers suggested that the hydroxyl group of the ultimate 3′-terminal nucleotide of polioviron RNA served as a primer for synthesis of negative-strand RNA (25). Andrews and Baltimore proposed that the host factor is a terminal uridylyl transferase which adds 3 to 4 uridylyl residues to the 3′ OH of the ultimate 3′-terminal nucleotide of poliovirion RNA (2). This short stretch of oligo(U) then snaps back on the poly(A) tail of virion RNA, and this structure serves as the template-primer for the synthesis of negative-strand RNA (2). These researchers, however, did not present any results showing in vitro synthesis of dimeric RNA molecules with poliovirus replicase and purified terminal uridylyl transferase (1). Unlike Andrews and Baltimore, who purified a host factor (terminal uridylyl transferase) from S100 fractions from HeLa cells, we reported purification of host factor from ribosomal salt wash fractions and showed that highly purified preparations of host factor containing a 67-kilodalton protein display double-stranded RNA-dependent protein kinase activity (17). Despite several attempts, we were unable to detect any terminal uridylyl transferase activity in our purified host factor preparations.

Whereas the results of Young et al. (25) could be explained by the mechanism proposed by Andrews and Baltimore (2), the view that newly synthesized negative-strand RNA is covalently linked to the exact 3′ end of input template RNA has never been demonstrated and is also clouded by two observations. (i) Lubinski et al. found that partially purified replicase containing a nuclease activity nicks the template RNA randomly, and the nicks made at or near a hairpin could produce a snap-back structure, the 3′ OH of which could be used as a primer to copy the template RNA (16). If a nick near the 3′ end of the template RNA is elongated by the replicase, the product appears to have been generated by extension from the exact 3′ end of the intact template. This result has recently been confirmed by Hey et al., who showed that preincubation of template RNA with crude host factor preparations modifies the RNA by the action of a contaminating nuclease. The modified RNA template, after reisolation by phenol extraction, could be elongated by the purified replicase alone to form apparently dimeric RNA molecules (14). These researchers also showed that the modified templates did not contain a 3′-terminal poly(A) tail, indicating that cleavage of the template occurred at a site before the start of the poly(A) tail. (ii) Hey et al. showed that synthesis of dimeric RNA products from a poliovirus RNA template was not at all affected by prior oxidation of the 3′ hydroxyl group of the ultimate 3′ nucleotide of poliovirion RNA. This result directly contradicts the view that the in

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vitro synthesized dimeric RNA results from covalent linkage between the exact 3′ nucleotide of template RNA and the newly synthesized negative-strand RNA.

An interesting observation by Lubinski et al. (16) and Hey et al. (13) was that the in vitro synthesized dimeric RNA products from a poliovirion RNA template consisted of both labeled positive and negative strands of RNA. To explain the synthesis of RNAs of both polarities, Lubinski et al. proposed the following model. Once initiated at or near the 3′ end of the poliovirion RNA template, the replicase copies the positive strand and, when it reaches the 5′ end of the template, the newly synthesized negative-strand RNA snaps back on itself and primes the synthesis of new positive strands of RNA (16). The strategy we used to study this unusual property of the viral replicase is to make the enzyme elongate an oligo(U) primer hybridized to the poly(A) tail of poliovirion RNA and ask whether the newly synthesized minus-strand RNA could snap back on itself to prime synthesis of a new positive strand. We show here that a highly purified preparation of poliovirus replicase is able to synthesize a covalently linked dimeric RNA molecule in vitro from a poliovirion RNA template. The reaction is absolutely dependent on added oligo(U). Filter hybridization studies suggested that the in vitro synthesized dimeric product consists of RNAs of both plus and minus polarities. We also show that a 5′ [^{32}P]oligo(U) primer can be elongated to RNA molecules of twice the size of the template. Our results also suggest that partially purified preparations of poliovirus replicase contain nuclease activity which can nick the dimeric RNA product, presumably at the single-stranded loop, where positive- and negative-strand RNAs are linked covalently to each other. This converts the dimeric RNA to template size RNA products. The highly purified replicase lacks this nuclease activity, thereby allowing detection of dimeric RNAs synthesized in an oligo(U)-dependent fashion from a poliovirion RNA template.

MATERIALS AND METHODS

All chemicals, unless specifically stated, were purchased from Sigma Chemical Co., St. Louis, Mo. Unlabeled nucleotides were obtained from Calbiochem-Behring, La Jolla, Calif. Poly(U)-Sepharose 4B was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Phosphocellulose was purchased from Whatman, Inc., Clifton, N.J. All radioisotopes were purchased from New England Nuclear Corp., Boston, Mass.

Purification of poliovirus replicase. Poliovirus replicase was purified by phosphocellulose chromatography (fraction II) as previously described (8). Fraction II replicase was further purified through poly(U)-Sepharose 4B by step elution with 0.25 M KCl (fraction III) (8, 9). Further purification of fraction III replicase involved two passages through poly(U)-Sepharose 4B, followed by elution of the enzyme with a 50 to 450 mM KCl gradient (step IV) from column 1 and elution with a 50 to 250 mM KCl gradient (step V) from column 2. Peak fractions containing poly(U) polymerase activity were pooled and concentrated with solid sucrose. The concentrated enzyme was dialyzed overnight and stored at −70°C (fraction V) in small portions. Further purification of fraction V replicase was carried out by gel filtration of the enzyme through an ACA 44 column (fractionation range, 30,000 to 132,000). An ACA 44 column (1.5 by 30 cm) was equilibrated with buffer A (50 mM Tris hydrochloride [pH 8.0], 10% glycerol, 0.1% Nonidet P-40, 5 mM β-mercaptoethanol, 50 mM KCl). Fraction V replicase was loaded onto the column at a flow rate of 0.5 ml/min. The column was then developed with 100 ml of buffer A. Fractions of 1 ml were collected, and samples of different fractions were assayed for poly(A) · oligo(U)-dependent poly(U) polymerase activity. The polymerase activity was included in the column (voided volume, 10 ml) and was eluted between fractions 20 and 30 (i.e., 20 to 30 ml). Fractions containing poly(U) polymerase activity were pooled, concentrated, and dialyzed against buffer A (fraction VI). The enzyme was stored at −70°C in small portions. Poliovirion RNA was prepared as previously described (22).

In vitro RNA synthesis with replicase and methylmercuric hydroxide gel electrophoresis. The standard reaction mixture for template-dependent replicase activity contained the following in 50 μl: 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 8.0), 5 mM magnesium acetate, 4 mM dithiothreitol, 10 μg of actinomycin D per ml, 30 mM KCl, 20 μM zinc chloride, 0.2 mM each ATP, GTP, and CTP, 1 to 2 μCi of [α-^{32}P]UTP, 5 μM unlabeled UTP, 1 μg of template RNA, 0.5 μg of fraction V replicase. In some experiments, fraction V replicase was replaced by 0.2 μg of fraction VI replicase. All reactions contained 100 ng of oligo(U) per ml. Incubation was for 30 to 60 min at 30°C. The RNA products were precipitated with trichloroacetic acid, collected on membrane filters, and counted in 5 ml of Brays solution. For analysis of the in vitro product, the phenol-extracted, ethanol-precipitated labeled RNA was suspended in diethylpyrocarbonate-treated water, denatured with 15 mM methylmercuric hydroxide, and analyzed on a 1.5% agarose gel containing 15 mM methylmercuric hydroxide as described by Bailey and Davidson (3).

RNA filter hybridization and two-dimensional gel electrophoresis. RNA filter hybridization experiments were as described by Zinn et al. (26). The full-length poliovirus minus-strand RNA used in hybridization experiments was prepared as previously described (15). Two-dimensional gel analysis of proteins was performed as described by O'Farrell (19). The first dimension was an isoelectric focusing gel in the pH range of 4 to 8. The second dimension was a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel. After separation of proteins in the two dimensions, the gel was dried and autoradiographed.

5′-End labeling of oligo(U). Oligo(U) (2.5 μg) was labeled in 100 μl of buffer containing 50 mM Tris hydrochloride (pH 9.0), 10 mM $MgCl_2$, 5 mM dithiothreitol, 200 μCi of [γ-^{32}P]ATP (specific activity, 5,000 Ci/mmol), and 25 U of T4 polynucleotide kinase (New England BioLabs, Beverly, Mass.). Incubation was at 37°C for 30 min. The reaction mixture was then diluted with 400 μl of 0.1 M Tris hydrochloride (pH 7.7)–10 mM triethylamine–1 mM EDTA and applied to a preequilibrated NENSORB 20 column (New England Nuclear). The column was washed thoroughly, and oligo(U) was eluted in approximately 400 μl of 50% methanol. The sample was evaporated to dryness, suspended in diethylpyrocarbonate-treated, deionized, distilled water, and then applied to a preequilibrated DEAE-52 (Whatman) column. After the column was washed, labeled oligo(U) was eluted with a 0 to 3 M sodium acetate (pH 5.1) gradient. Most of the trichloroacetic acid-precipitable counts eluted between 1.5 and 2.5 M salt. The end-labeled oligo(U) was recovered by dilution of the salt to 0.5 M and addition of $MgCl_2$ to 1 mM, followed by precipitation with 2.5 volumes of absolute ethanol. The end-labeled oligo(U) was collected by centrifugation and suspended in diethylpyrocarbonate-treated water. It was used at a concentration of 100 ng/ml in RNA synthesis.

RESULTS

Oligo(U)-dependent synthesis of dimeric RNAs by poliovirus replicase. Previous results from a number of laboratories showed that the poliovirus RNA template could be copied by poliovirus replicase in the presence of oligo(U) primer to generate template length minus-strand RNA products (6, 7, 13, 23). In the absence of oligo(U) primer, viral RNA polymerase was completely inactive. It was also shown that 5′ [^{32}P]oligo(U) could be elongated by viral replicase to template length products in the presence of all four unlabeled nucleoside triphosphates (6, 24). The assay conditions used for oligo(U)-primed synthesis of full-length minus-strand RNA was 60 min of incubation after mixing of all of the components required for RNA synthesis. Because our previous results suggested that, in the presence of poliovirus replicase, a newly synthesized negative-strand RNA could snap back on itself, generating a template-primer for the synthesis of new positive-strand RNA (16), we reexamined oligo(U)-primed RNA synthesis by poliovirus replicase. When a template-dependent preparation of poliovirus replicase was incubated with virion RNA, oligo(U), [α-^{32}P]UTP, and three other unlabeled nucleoside triphosphates at 30°C for 60 min and the labeled RNA product was analyzed by denaturing agarose gel electrophoresis, the predominant product was full-length RNA (Fig. 1, lane 3). When the reaction was stopped at 30 min of synthesis, some of the labeled products migrated at a position corresponding to twice the length of template RNA (lane 2). The labeled, in vitro synthesized, high-molecular-weight RNA product cosedimented with double-stranded RNA (replicative form) isolated from poliovirus-infected cells during density gradient centrifugation under nondenaturing conditions (data not shown). A template length product was also observed at 30 min; however, the intensity of the 35S band was much less than that observed at 60 min. In the absence of added oligo(U), no labeled product could be detected. These results suggested that the poliovirus replicase was able to synthesize covalently linked dimeric RNA molecules in the presence of an oligo(U) primer and that longer incubation of these products at 30°C allowed cleavage of the larger RNA molecules to template length products.

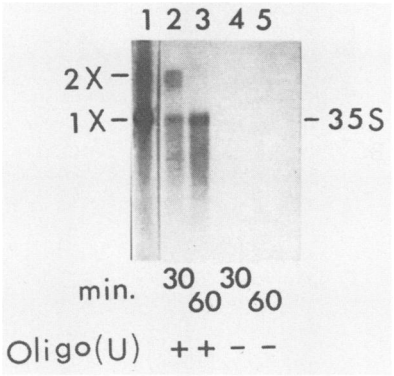

FIG. 1. In vitro synthesis of dimeric RNA by poliovirus replicase. Fraction V poliovirus replicase (0.5 μg) was used for synthesis of labeled RNA from the poliovirion RNA template in the presence of oligo(U) as described in Materials and Methods. Labeled RNAs synthesized in vitro or isolated in vivo were denatured by 15 mM methylmercury and analyzed by agarose gel electrophoresis in the presence of 15 mM methylmercury. Lanes: 1, in vivo labeled RNA isolated from poliovirus-infected cells; 2, in vitro synthesized RNA with oligo(U), 30 min of incubation; 3, same as lane 2, except that RNA synthesis was stopped after 60 min; 4, same as lane 2, except that oligo(U) was omitted from the reaction; 5, same as 3, except that oligo(U) was omitted from the reaction. Monomeric and dimeric RNAs are indicated by 1× and 2×, respectively.

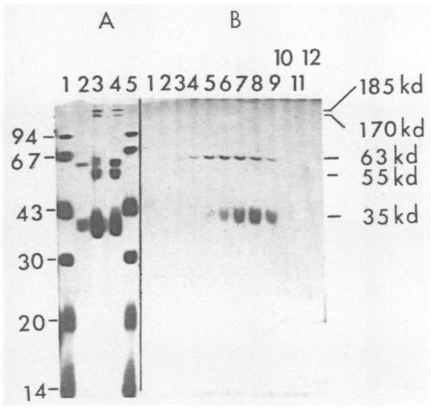

FIG. 2. Purification of poliovirus replicase. Poliovirus replicase was purified from 5×10^9 HeLa cells infected with poliovirus as described in Materials and Methods. Fraction V and VI replicase preparations were analyzed by SDS-polyacrylamide gel electrophoresis followed by silver staining of the gel. (A) Lanes: 1, molecular weight marker proteins; 2, pooled and concentrated fraction VI replicase; 3, pooled and concentrated fraction V replicase; 4, another batch of pooled and concentrated fraction V replicase; 5, molecular weight markers. (B) Analysis of fractions containing polymerase activity from an ACA 44 column. Lanes 1 to 12 contained fractions no. 19 to 30, respectively, from the ACA 44 column. The numbers to the left and right indicate molecular weight in thousands.

Further purification of poliovirus replicase. The replicase preparation (step V) used in the experiments shown in Fig. 1 was purified from a cytoplasmic extract (step I) from poliovirus-infected HeLa cells through phosphocellulose (step II) and poly(U)-Sepharose (batch elution, step III) chromatography. The step III enzyme was further purified by two additional passages through poly(U)-Sepharose, and each time the enzyme was eluted with a shallower salt gradient (50 to 450 mM KCl gradient in step IV and 50 to 250 mM KCl gradient in step V). This replicase preparation contained five polypeptides as visualized by silver staining of SDS-polyacrylamide gels (Fig. 2A, lanes 3 and 4). The approximate molecular weights of these polypeptides were 185,000, 170,000, 63,000, 55,000, and 35,000. When step V replicase was prepared from cells labeled with [^{35}S]methionine at 3.5 h postinfection, the condition that allows labeling of only viral proteins, two virus-coded polypeptides, 3D^{pol} and 3CD, were detected in the preparation (Fig. 3A, lane 1). Step V replicase, therefore, contained two viral polypeptides and at least four cellular proteins.

The viral RNA polymerase was further purified by passage through a gel filtration column (ACA 44). Most of the polymerase activity was included in the column and eluted at an approximate molecular weight of 100,000. Analysis of fractions containing viral RNA polymerase activity showed two bands stainable by silver, having approximate molecular weights of 63,000 and 35,000 (Fig. 2B). It was clear from the results (Fig. 2B) that chromatography of the polymerase on ACA 44 resulted in removal of 185,000-, 170,000-, and 55,000-molecular-weight proteins. When the active fractions from this column were pooled and concentrated (step VI) and the proteins were analyzed by SDS-polyacrylamide gel

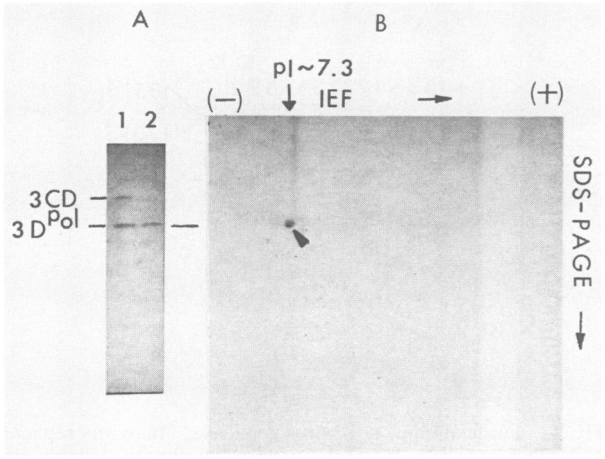

FIG. 3. (A) Analysis of [^{35}S]methionine-labeled viral proteins in fraction V and VI replicase preparations by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Lanes: 1, fraction V replicase; 2, fraction VI replicase. (B) Analysis of [^{35}S]methionine-labeled viral proteins in fraction VI replicase by two-dimensional gel electrophoresis. The arrowhead indicates the position of p63 ($3D^{pol}$). IEF, Isoelectric focusing.

electrophoresis, only two bands of approximate molecular weights 63,000 and 35,000 were detected (Fig. 2A, lane 2). Analysis of [^{35}S]methionine-labeled viral proteins in a step VI enzyme preparation by either one- or two-dimensional gel electrophoresis showed that it contained only one viral polypeptide (p63) (Fig. 3A, lane 2, and Fig. 3B). Silver staining of the two-dimensional gel showed that the 35,000-M_r protein migrated with heterogeneous pIs of 4.5 to 5 (data not shown). It was clear from these results that the most-purified preparations of poliovirus replicase contained two polypeptides, one of which was virus coded and the other of which was most probably of cellular origin.

Highly purified poliovirus replicase synthesizes dimeric RNA products. When fractions from the ACA 44 column (step VI) were analyzed for their ability to support poliovirion RNA-dependent RNA synthesis, the most prominent product of these reactions was twice the size of the template (Fig. 4A). Synthesis of RNA products by these fractions was completely dependent on added oligo(U) primer (Fig. 4B). It should be noted that the product RNA consisted of two closely spaced bands, of which the slower-migrating one was approximately twice the length of the template. The products synthesized by the fractions corresponding to the ascending and trailing peaks of polymerase activity consisted mainly of the faster-migrating RNA (Fig. 4A, arrow), whereas the peak fractions synthesized both dimeric RNA and the smaller product.

To determine whether a functional difference existed between step V and VI enzymes, the kinetics of RNA synthesis were determined by using these two preparations. Step V and VI replicase preparations were incubated with poliovirion RNA and nucleoside triphosphates in the absence or presence of oligo(U) primer under RNA synthesis assay conditions. Reactions were stopped at various times after the start of synthesis, and labeled products were analyzed by methylmercury agarose gel electrophoresis. Both preparations of enzyme were absolutely dependent on added oligo(U) and synthesized smaller RNA products at early time points (Fig. 5). The size of newly synthesized RNA increased with time, and the size of the product reached near-template length at approximately 20 to 25 min of synthesis, depending on the preparation of enzyme used in these experiments. Synthesis of products of approximately twice the length of the template occurred at 25 to 30 min of RNA synthesis. With step V enzyme, some template size RNA product appeared at 30 min, and at 40 min of synthesis, most of the larger RNA molecules disappeared, with a concomitant increase in the intensity of template size product. The pattern of RNA synthesis catalyzed by step VI enzyme was quite different from that catalyzed by step V enzyme. The predominant RNA product at late time points consisted mainly of dimeric RNA molecules. At 40 min of synthesis, no template size RNA could be detected with step VI enzyme. Further incubation of this reaction for up to 60 min did not reduce the size of the product (data not shown). These results suggest that step V enzyme is contaminated with a nuclease activity which is capable of cleaving dimer size RNA molecules to template length products. This nuclease activity is purified away by gel filtration of step V polymerase, and thus the dimeric products synthesized by step VI polymerase remain intact.

In vitro synthesized RNA consists of both plus and minus strands. To determine whether dimeric RNAs synthesized by step VI replicase in the presence of oligo(U) consists of both plus and minus strands, filter hybridization studies were performed. [^{32}P]UMP-labeled dimeric RNA was synthesized in vitro by using step VI replicase and oligo(U) from the poliovirion RNA template. The reaction was stopped at 40 min of synthesis, and labeled RNA products were extracted with phenol and precipitated with ethanol. The product RNA was then digested with S1 nuclease. Labeled RNA in solu-

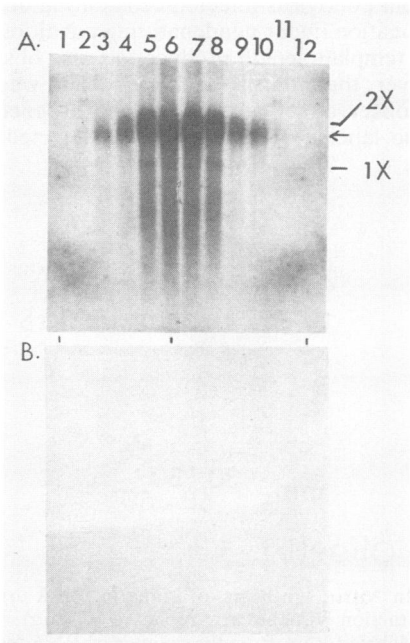

FIG. 4. Oligo(U)-primed synthesis of RNA by different fractions from an ACA 44 column. Portions (10 μl each) of various fractions from an ACA 44 column were assayed for poliovirion RNA-dependent RNA synthesis in the presence (A) or absence (B) of added oligo(U) primer at 30°C for 60 min. Labeled products were analyzed by methylmercury agarose gel electrophoresis. Lanes 1 to 12, containing fractions no. 19 to 30, respectively, from the ACA 44 column (as in Fig. 2B) were used for RNA synthesis. Monomeric and dimeric RNAs are indicated by 1× and 2×, respectively. The arrow indicates faster-migrating RNA species, as discussed in the text.

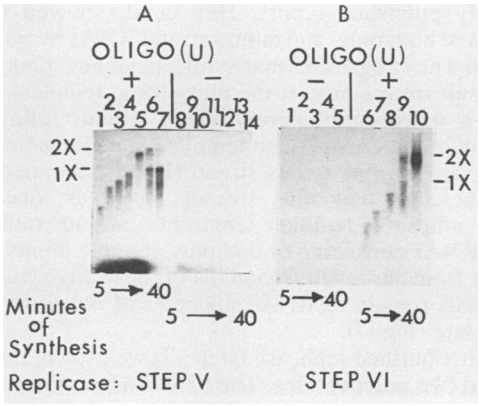

FIG. 5. Kinetics of RNA synthesis by fraction V and VI replicase preparations. Poliovirion RNA-directed RNA synthesis in the presence or absence of oligo(U) primer was performed by using fraction V and VI replicase preparations as described in Materials and Methods. Reactions were stopped at different times of synthesis. Labeled products, after denaturation with 15 mM methylmercury, were analyzed by agarose gel electrophoresis in the presence of 15 mM methylmercury. (A) Kinetics with fraction V replicase. Synthesis times (lanes): 1 and 8, 5 min; 2 and 9, 10 min; 3 and 10, 15 min; 4 and 11, 20 min; 5 and 12, 25 min; 6 and 13, 30 min; 7 and 14, 40 min. (B) Kinetics with fraction VI enzyme. Synthesis times (lanes): 1 and 6, 5 min; 2 and 7, 10 min; 3 and 8, 15 min; 4 and 9, 25 min; 5 and 10, 40 min. Monomeric and dimeric RNAs are indicated by 1× and 2×, respectively.

tion (after phenol extraction and ethanol precipitation) was allowed to hybridize with unlabeled poliovirion RNA, minus-strand RNA, or RNA immobilized to nitrocellulose filters. It was clear from the results (Fig. 6) that the RNA products synthesized during the first 10 min consisted mainly of minus strands. At 20 min of synthesis, although most of the newly synthesized RNA was of minus polarity, some plus-strand synthesis was evident. At later time points,

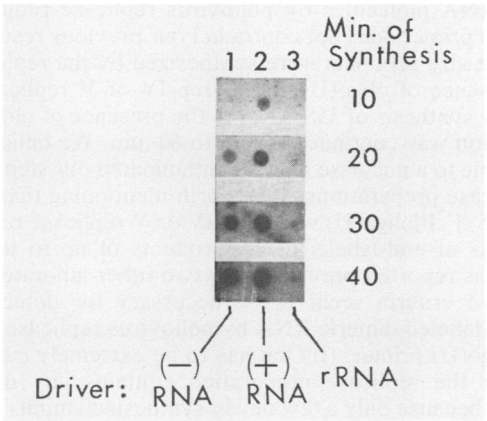

FIG. 6. Dot blot hybridization of labeled RNA products to various unlabeled RNAs. Approximately 200 ng each of unlabeled minus-strand RNA (lane 1), plus-strand RNA (lane 2), and rRNA (lane 3) was spotted on different filter papers. [^{32}P]UMP-labeled RNA products synthesized in vitro from the poliovirion RNA template by fraction VI replicase for various times (10, 20, 30, and 40 min), as indicated, were digested with S1 nuclease. The products were diluted with prehybridization buffer, and the labeled products in solutions were allowed to hybridize with the spotted unlabeled RNAs by incubating the filter papers with the appropriate labeled solutions as described in Materials and Methods.

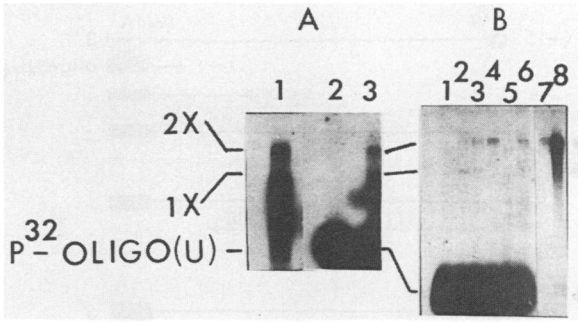

FIG. 7. Extension of 5′ [^{32}P]oligo(U) to product RNA twice the size of template RNA. 5′ [^{32}P]oligo(U) was prepared by the procedure described in Materials and Methods. End-labeled oligo(U) was incubated with fraction VI replicase and poliovirion RNA template in the presence of all four unlabeled nucleoside triphosphates. ^{32}P-end-labeled RNA products were analyzed by methylmercury agarose gel electrophoresis. End-labeled RNA products synthesized in the presence of all four unlabeled nucleoside triphosphates and 5′ [^{32}P]oligo(U) were pooled from four 50-μl reaction mixtures. The positions of migration of dimeric and monomeric RNAs, respectively, are indicated by 1× and 2×. (A) Lanes: 1, products synthesized by step VI replicase (batch I) in the presence of unlabeled oligo(U), [^{32}P]UTP, and three other unlabeled nucleoside triphosphates; 2, products synthesized in the presence of 5′ [^{32}P]oligo(U) and all four unlabeled nucleoside triphosphates in the absence of added replicase; 3, same as in lane 2, except that step VI replicase was added during RNA synthesis. (B) Kinetics of RNA synthesis with 5′-end-labeled oligo(U). All reactions contained 5′ [^{32}P]oligo(U), four unlabeled nucleoside triphosphates (except lane 5), and step VI replicase. Synthesis times (lanes): 1, 5 min; 2, 20 min; 3, 25 min; 4, 30 min; 5, same as lane 4, but the four nucleoside triphosphates were omitted from the reaction; 6, same as lane 4; 7 and 8, products synthesized at 30 min with [α-^{32}P]UTP plus three other unlabeled nucleoside triphosphates in the presence of unlabeled oligo(U). Lane 7 contained approximately 1/50 of the amount of [α-^{32}P]UTP-labeled product analyzed in lane 8.

however, significant amounts of RNAs of plus polarity were detected.

In vitro synthesized dimeric RNA is linked to oligo(U) primer. To determine whether dimeric RNA synthesized in vitro by step VI polymerase is physically linked to the oligo(U) primer, poliovirion RNA-directed RNA synthesis was performed in the presence of 5′ [^{32}P]oligo(U) and all four unlabeled nucleoside triphosphates. End-labeled RNA products were analyzed by methylmercury agarose gel electrophoresis. Clearly, end-labeled oligo(U) could be extended to RNA molecules having twice the length of the template (Fig. 7). Omission of replicase (Fig. 7A, lane 2) or nucleoside triphosphates (Fig. 7B, lane 5) from the reactions resulted in complete loss of synthesis of end-labeled RNA products. Formation of [^{32}P]oligo(U)-labeled RNA was time dependent (Fig. 7B). At earlier time points, synthesis of template size products was detected (Fig. 7B, lanes 2 and 3). At later time points, however, most of the product was twice the length of the template (Fig. 7B, lanes 4 and 6). Two different preparations of step VI replicase were used in the experiments shown in Fig. 7A (batch I replicase) and B (batch II replicase). Clearly, batch II replicase was more active than batch I replicase in that almost all of the products synthesized by batch II replicase was twice the size of the template, whereas those synthesized by batch I replicase contained both monomeric and dimeric RNAs (Fig. 7B, lanes 1 and 3, and B, lanes 4 and 8). Incubation of dimeric RNA products, synthesized by batch II replicase, with batch I polymerase in

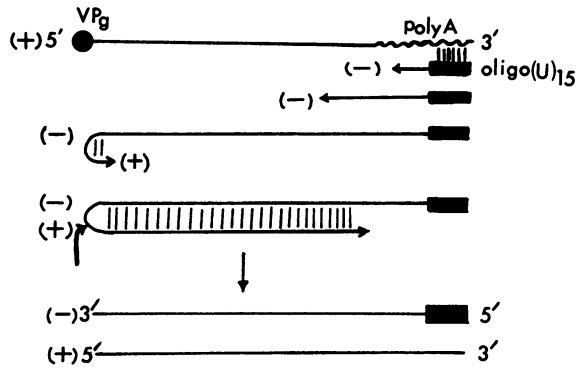

FIG. 8. A model for oligo(U)-directed synthesis of plus and minus strands by poliovirus replicase. Oligo(U) hybridized to the poly(A) tail of poliovirion RNA is elongated by poliovirus replicase. The newly synthesized minus strand snaps back when replicase reaches the 5′ end of the template. The snapped-back RNA is then elongated by replicase, copying the newly synthesized minus strand. A nuclease activity present in fraction V replicase nicks the single-stranded loop, generating template length RNA molecules of plus and minus polarities.

the absence of added nucleoside triphosphates showed that batch I polymerase contained no nuclease activity. We concluded, therefore, that batch I polymerase was unable to utilize minus strands quantitatively for synthesis of new plus strands. These results suggest that synthesis of covalently linked dimeric RNA by step VI replicase occurs by snap back of newly synthesized minus-strand RNA, followed by elongation of snapped-back RNA to generate new plus strands.

DISCUSSION

We have shown that a highly purified preparation of poliovirus replicase, consisting of two polypeptides of M_rs 63,000 and 35,000, was able to copy poliovirion RNA to generate RNA products of twice the size of the input template RNA. The dimeric RNA product appeared to be due to formation of a covalent linkage between two complementary strands of RNA. It should be noted that the mobility of these dimeric RNA products was unaffected by phenol extraction (Fig. 1) or proteinase K digestion (data not shown).

Three lines of evidence suggest that these dimeric molecules are produced by snap back of newly synthesized minus strands, followed by elongation of the snapped-back template to generate plus strands (Fig. 8). (i) Synthesis of dimeric RNAs is absolutely dependent on added oligo(U) primer. Since no radioactive products could be detected in the absence of oligo(U), the possibility that dimeric RNA synthesis is due to extension of the template following nucleolytic cleavage of the template at or near a hairpin, which generates a self-priming template (16), is highly unlikely. Clearly, then, the dimeric product we observed does not represent newly synthesized RNA covalently linked to template RNA. (ii) Dot blot hybridization studies showed that both plus and minus strands of RNA are synthesized during the in vitro reaction. Perhaps the most convincing evidence that plus-strand synthesis occurs following snap back of newly synthesized minus-strand RNA comes from the experiments with 5′ [^{32}P]oligo(U) primer. It is clear that end-labeled oligo(U) primer can be extended to RNA products having twice the size of the template (Fig. 7). In a previously published report, Hey et al. showed in vitro synthesis of both plus- and minus-strand RNAs by poliovirus replicase. They suggested that synthesis of new plus strands could result from a nick at the plus-strand template hybridized to a newly synthesized minus strand, followed by peeling off of the downstream template and elongation at the nick site copying the minus strand (13). In this mechanism for synthesis of new plus strands, however, one would expect synthesis of template length plus strands rather than dimeric RNAs consisting of one plus and one minus strand. It is clear from our results that most of the newly synthesized plus strands remain as RNA molecules of twice the size of the template (Fig. 7).

The most-purified replicase (step VI) we used in this study contained two polypeptides. The 63,000-M_r polypeptide was virus coded, whereas the 35,000-M_r polypeptide appeared to be of cellular origin (Fig. 2 and 3). The molecular weight and pI of the poliovirus-specific RNA-dependent RNA polymerase were in agreement with a previously published report (23). The 35,000-M_r protein appeared to be complexed to p63 (Fig. 2B), and our repeated attempts to purify this protein away from polymerase failed. This protein (M_r, 35,000) may be required for the snap-back reaction to occur. In this context, it should be pointed out that other investigators previously showed synthesis of only template length products by the poliovirus replicase in the presence of oligo(U) (13, 25). The replicase used in those experiments were prepared by using a different protocol than that reported here. It is possible that their replicase preparations lacked one or more proteins (the 35,000-M_r protein?) responsible for the snap back of minus-strand RNA.

Step V polymerase contained a nuclease which was apparently capable of nicking the dimeric RNA to produce template length molecules (Fig. 5). Since most of the nuclease activity was removed by further purification of step V polymerase by gel filtration on an ACA 44 column, a step which also resulted in removal of the M_r 185,000, 170,000, and 55,000 polypeptides, any one or more of these polypeptides may be responsible for the nuclease activity.

The observation that synthesis of covalently linked dimeric RNA molecules by poliovirus replicase requires an oligo(U) primer does not contradict our previous results that template size products were synthesized by the replicase in the presence of oligo(U). With step IV or V replicase, we still saw synthesis of 35S RNA in the presence of oligo(U) if incubation was continued for up to 60 min. We believe that it was due to a nuclease which contaminated our step IV and V replicase preparations. It is worth mentioning that extension of 5′ [^{32}P]oligo(U) with step IV or V replicase results in synthesis of end-labeled RNA products of up to template length, as reported previously by two other laboratories (6, 24). Two criteria seem to be necessary for detection of ^{32}P-end-labeled dimeric RNA by poliovirus replicase with 5′ [^{32}P]oligo(U) primer. (i) One has to be extremely careful of whether the replicase preparation contains any nuclease activity because only a few newly synthesized minus strands are used for plus-strand synthesis. So any contamination of replicase by nuclease would minimize the possibility of detecting extended molecules. (ii) The replicase would have to be active enough to catalyze snap back of newly synthesized minus strands.

It is not known at present whether synthesis of dimeric RNA by the mechanism outlined in Fig. 8 has any physiological significance. It should be noted that covalently linked dimeric RNA molecules have been detected in picornavirus-infected cells (21, 25). It is tempting to speculate that

synthesis of plus-strand RNA by this mechanism may represent a step by which poliovirus mRNA is produced. It is known that viral mRNA isolated from virus-specific polyribosomes does not contain the 5'-terminal covalently linked protein (VPg) but instead has a 5'-terminal phosphate (12). The plus strand generated by nucleolytic cleavage of the dimeric RNA may contain a phosphate at its 5' end. Clearly, analysis of the 5'-end sequences of newly synthesized plus-strand RNAs would be necessary before any valid conclusion can be drawn. It is worth mentioning in this context that plus-strand RNAs synthesized in vitro from minus-strand RNA templates with replicase preparations containing the putative nuclease were found to be infectious in in vitro transfection assays (15). If one assumes that synthesis of plus-strand RNA from a minus-strand template occurs by a mechanism such as that illustrated in Fig. 8, the in vitro synthesized product must have been cut at or near the exact junction of the plus and minus strands to yield infectious plus strands.

The results presented here clearly demonstrate that the ability to synthesize covalently linked dimeric RNA products reside in the replicase preparation containing two polypeptides. In view of this result, extreme caution must be exercised before considering any model for synthesis of minus-strand RNA from a poliovirion RNA template with a replicase-host factor combination. In future, it would be necessary to determine the nature of the products synthesized in a host-catalyzed reaction by using step VI replicase.

Reports from two laboratories suggested that the 3' end of the plus-strand RNA template could snap back and self-prime synthesis of new minus strands in vitro (2, 25). Our previous results (16) and those from the laboratory of E. Ehrenfeld (14), however, showed that this could be due to cleavage of template RNA at or near a hairpin near its 3' end, followed by extension by the replicase of the 3' OH generated by nucleolytic cleavage. The results reported here are not consistent with snap back of the 3' end of template RNA since dimeric RNA molecules are only observed in the presence of oligo(U) primer. Additionally, snap back of the 3' end of template RNA does not explain why both labeled plus and minus strands are synthesized in vitro by the replicase in the presence of oligo(U) primer. Finally, if indeed a mechanism similar to the one outlined in Fig. 8 operates in poliovirus-infected cells, it would still be consistent with a VPg-priming model for the initiation of viral RNA synthesis.

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